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Brief communication

Survival of mint shoot tips after exposure to cryoprotectant solution components ☆

Gayle M. Volk *, Jackie L. Harris, Kate E. Rotindo

National Center for Genetic Resources Preservation, United States Department of Agriculture, 1111 S. Mason St., Ft. Collins, CO 80521, USA

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Abstract

Many plant species can be cryopreserved by treating shoot tips with complex cryoprotectant solutions before rapidly cooling them to liquid nitrogen temperatures. Plant vitrification solution 2 (PVS2), a commonly selected cryoprotectant, can be lethal with extended exposure times. To determine potentially toxic combinations, we have exposed mint shoot tips to one-, two-, three-, and four-component solutions of PVS2 chemicals (30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide, and 0.4 M sucrose) at 0 and 22 °C. Overall, solution exposures at 22 °C were more damaging than exposures at 0 °C. Solutions with glycerol, particularly in combination with ethylene glycol and dimethyl sulfoxide, were also damaging. Cryoprotectant solutions PGluD (10% PEG8000, 10% glucose, and 10% dimethyl sulfoxide) and PVS3 (50% glycerol, 50% sucrose) were less damaging than PVS2 at 22 °C. When plant cryoprotectants are characterized on a toxicological and biophysical basis, less damaging cryoprotectant solutions could be developed.

Keywords: Dimethyl sulfoxide; Ethylene glycol; Glycerol; Shoot tips; Vitrification; Cryopreservation; *Mentha*

Plant and animal repositories maintain many cell types and tissues at liquid nitrogen (LN) temperatures for extended lengths of time. Most biological materials cannot survive these conditions without the addition of cryoprotective solutions. To prevent cellular damage, cryoprotectant solutions both dehydrate and penetrate cells to stabilize proteins and membranes; however, the mode of action of each component may differ with cell type, species, temperature, and other solution components [2,3]. The process of exposing to cryoprotectants, cooling, and then warming explants from extreme temperatures can damage cell structure and impair physiological function [9]. Chemical toxicity can cause some of this damage [9].

Vitrification procedures, where shoot tips are treated with a highly concentrated solution of dehydrating and penetrating chemicals prior to a plunge into LN, can promote survival of cryo-exposed cells [1,2]. Successful use of plant vitrification

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* Corresponding author. Fax: +1 970 221 1427.
E-mail address: gvolk@lamar.colostate.edu (G.M. Volk).

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solution 2 (PVS2; 30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide, and 0.4 M sucrose in plant growth medium salts [8]) frequently involves a 20–30 min exposure to PVS2 at 0 °C [1]. Alternatively, exposure to plant vitrification solution 3 (PVS3; 50% sucrose, 50% glycerol [7]) may require up to 240 min at room temperature for successful shoot tip cryopreservation [4]. For plants, it is generally agreed that the cryoprotective solution PVS2 can be damaging [1]. PVS2 removes cellular water, changes cellular freezing characteristics, limits ice crystallization and has components that permeate cells [11]. Exposure time and conditions must be optimized to obtain sufficient protection from ice formation without killing cells from osmotic or chemical stress [1].

Two-step cooling methods treat explants with PGluD, which is less concentrated than PVS2. PGluD contains 10% polyethylene glycol 8000 (PEG8000), 10% glucose (glu), and 10% dimethyl sulfoxide [12]. Two-step cooling methods rely on freeze–dehydration over several hours as water is drawn out of the shoot tips during the slow cooling step of the procedure. The slow cooling rate (0.1–1 °C min^{-1}) to −30 or −40 °C prior to a plunge into LN provides adequate time for the dehydration, and alleviates the necessity of using more concentrated cryoprotectant solutions [1,12].

The purpose of this study was to quantify the survival of mint shoot tips after exposure to cryoprotectant solution components and to identify particularly damaging combinations of cryoprotectant components. Mint shoot tips can be cryopreserved using PVS2 as a cryoprotectant with 50–90% regeneration, depending on species and treatment conditions [10].

Mentha × *piperita* L. cv. Todd Mitcham Peppermint (PI 557973) plants were maintained in vitro and propagated on MS basal mineral and vitamin medium [6] with 3% sucrose and 1% agar. Single node sections were cultured for 3 days in a growth room at 25 °C and 16 h days ($32 \pm 5\ \mu mol \cdot m^{-2} s^{-1}$). Shoot tips (1 mm^3) were excised from lateral buds that emerged from the single node sections. Shoot tips were floated in liquid MS medium with 0.3 M sucrose overnight at 22 °C under ambient conditions in the laminar flow hood and then exposed to cryoprotective solutions at either 22 or 0 °C. A minimum of 15 shoot tips were treated with sterile cryoprotective solutions in 4 cm Petri dishes for 0.5, 1, 2, 3, 4, or 6 h. Each experiment was performed at least twice. Solutions were removed and shoot tips were treated with 0.3 or 1.2 M sucrose for 20 min at 22 °C and plated onto medium containing MS salts and vitamins, 0.5 mg L^{-1} benzyladenine, 0.1 mg L^{-1} indole butyric acid, 3% sucrose, and 0.7% agar, pH 5.7. Shoot tips on plates were kept in the dark overnight, transferred to fresh medium after 1 day, and then returned to the growth room. The number of explants with true shoots was recorded for each treatment after 4 weeks of culture.

There were no significant differences in survival or regrowth percentages between shoot tips rinsed with 0.3 or 1.2 M sucrose for 20 min, so these treatments were pooled for statistical analyses. The duration of solution exposure time that resulted in 50% of the shoot tips elongating into shoots (LT50) was calculated for each replicate of a time series within an experiment (ED50 plus version 1.0; http://www.softlookup.com/display.asp?ID=2972&DID=4J58YURT). Analyses of variance (ANOVA) and means separation tests were performed using the JMP software package (SAS Institute, Cary, NY, USA).

For PVS2 studies, shoot tips were treated with one-, two-, three-, or four-component solutions of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide, and/or 0.4 M sucrose in water. At 0 °C, mint shoot tips had LT50s greater than 6 h when treated with solutions of ethylene glycol or dimethyl sulfoxide either with or without sucrose (Fig. 1). At 22 °C, LT50s were significantly reduced to less than 2.5 h when solutions contained both ethylene glycol and dimethyl sulfoxide.

Glycerol was less damaging when mint shoot tips were exposed at 0 °C compared to exposures at 22 °C. At 22 °C, any solution containing glycerol killed 50% of the shoot tips in less than 2 h. Solutions containing glycerol–ethylene glycol or glycerol–dimethyl sulfoxide resulted in LT50s of 1 h or less. Glycerol–sucrose, glycerol–ethylene glycol, glycerol–ethylene glycol–sucrose, and PVS2 had significantly higher LT50s at 0 °C compared to 22 °C (Fig. 1).

Ethylene glycol and dimethyl sulfoxide are permeable in shoot tips [9,11]. These solutions may exhibit surfactant effects on membrane permeability, increasing the diffusion of glycerol into cells [3]. While this may be desirable for effective cryoprotection, an increase in intracellular glycerol concentration may also be damaging. Cryoprotectant permeability is lower at 0 °C than at 22 °C [5] and some of the negative effects of glycerol were ameliorated by reduced temperature exposures.

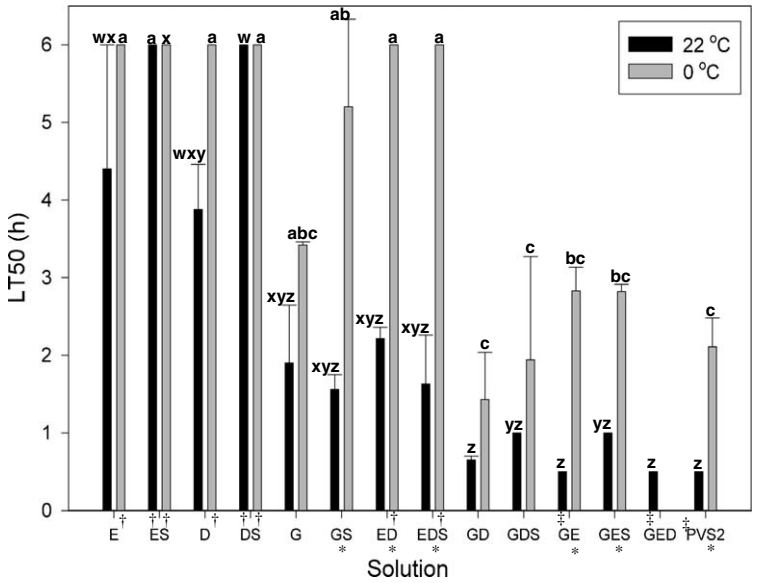

Fig. 1. LT50s (with standard errors) were calculated for mint explants treated with 30% glycerol (G), 15% ethylene glycol (E), 15% dimethyl sulfoxide (D), and 0.4 M sucrose (S) in one-, two-, three-, and four-component solutions. Letters denote significant differences using Tukey's Honestly Significantly Different means separation tests ($\alpha < 0.05$) for treatments conducted at 22 °C (a,b,c) and 0 °C (w,x,y,z). Asterisks represent significant differences between temperature treatments for a specific solution. Some solutions had LT50s greater than 6 h (†) or less than 30 min (‡). The 30% glycerol–15% ethylene glycol–15% dimethyl sulfoxide treatment was not performed at 0 °C.

PVS3, composed of sucrose and glycerol, is an alternative cryoprotectant used in some vitrification protocols. LT50s for mint shoot tips exposed to either the sucrose or glycerol components were calculated for mint shoot tips bathed in solutions at 22 °C. Mint exposed to 50% glycerol had an LT50 of less than 1 h at 22 °C, while exposure to 50% sucrose gave an LT50 of greater than 6 h. Overall, PVS3 treatment at 22 °C gave an LT50 of 2.5 ± 0.2 h (Table 1). Pretreatment of shoot tips with 2 M glycerol + 0.4 M sucrose for 20 min at 22 °C prior to PVS3 component exposure or a 1.2 M sucrose rinse for 20 min at 22 °C after PVS3 component exposure did not improve shoot tip survival (Table 1).

Our conclusion that glycerol was more damaging in the presence of ethylene glycol and dimethyl sulfoxide is supported by comparing LT50s for PVS2 and PVS3. PVS2, a solution that contains glycerol, ethylene glycol, dimethyl sulfoxide, and sucrose, has an LT50 of less than 1 h at 22 °C, while the LT50 of PVS3, containing just sucrose and glycerol (at higher concentrations than PVS2) is 2.5 ± 0.2 h at 22 °C. Effective cryopreservation with PVS3 often requires a longer soaking period than is necessary with PVS2, perhaps indicating a longer time needed to accumulate intracellular concentrations of glycerol sufficiently high for cryoprotection when ethylene glycol and dimethyl sulfoxide are not present.

Table 1
LT50 of PGluD and PVS3 cryoprotectant solutions and their components at 22 °C in mint shoot tips

PGluD components	LT50 (h)	PVS3 components	LT50 (h)
10% Me_2SO_4	>6	50% glycerol	<1
10% glucose	>6	50% glycerol/pretreatment/rinse	<1
10% PEG	>6	50% sucrose	>6
10% Me_2SO_4 + 10% glucose	>6	50% sucrose/pretreatment/rinse	>6
10% Me_2SO_4 + 10% PEG	>6	PVS3	2.5 ± 0.2
10% PEG + 10% glucose	>6		
10% PGluD	>6		

PVS3 components were also subjected to a 2 M glycerol + 0.4 M sucrose pretreatment for 20 min at 22 °C and a 1.2 M sucrose rinse for 20 min at 22 °C where indicated by pretreatment/rinse.

Two-step cooling methods frequently use PGluD, a more dilute and less damaging cryoprotectant solution than PVS2. Shoot tips were treated with PGluD solutions containing 10% dimethyl sulfoxide (w/v), 10% glucose (w/v), and/or 10% PEG8000 (w/v) in water in one-, two-, or three-component solutions. Treatments with these solutions had LT50s greater than 6 h (Table 1). Our data demonstrate that the PGluD solution commonly used in two-step cooling procedures caused minimal lethal damage in shoot tips.

The experiments presented here suggest that glycerol is a major component causing damage in mint shoot tips. Some of these negative effects can be alleviated by performing treatments at reduced temperatures. Alternative cryopreservation solutions that cause less damage may yield more uniform survival after cryo-treatments within and among plant species.

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